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(54) Title: SAPONIN ADJUVANT COMPOSITION

(57) Abstract

An adjuvant composition for stimulating an effective immune response to an antigenic substance when co-administered to an animal with said antigenic substance, comprising: (a) a saponin with immune stimulating activity; (b) a polycationic polyelectrolyte with immune stimulating activity; and (c) an immunoadjuvant oil.

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SAPONIN ADJUVANT COMPOSITION

TECHNICAL FIELD

This invention relates to adjuvant compositions for stimulating an immune response to an antigenic substance when co-administered to an animal with said antigenic substance, and to vaccines containing said adjuvant composition.

BACKGROUND ART

Vaccination against disease has a long history. In general terms the technique involves injection of an antigenic substance, or antigen, into an animal whereby the presence of the antigenic substance generates an immune response in the animal. Classical vaccination techniques involve the injection of killed organisms but more recently vaccines comprising attenuated live organisms or antigenic components of an organism have been developed. It is frequently found with killed vaccines and, more particularly, with vaccines comprising a component of an organism that the immune response is substantially less than the response to natural infection. However, the effectiveness of such vaccines can be considerably enhanced by the co-administration of a suitable adjuvant composition with the antigenic substance. Adjuvants, while not necessarily being antigenic themselves, potentiate or enhance an animal's immune response to the antigenic substance with which it is challenged. There are many adjuvants known and used but there is an ongoing need to identify new and effective adjuvants which are inexpensive, which produce minimal injection site irritation and discomfort and which are widely applicable and effective.

A common formulation for vaccines is to present the antigen(s) in an aluminium hydroxide gel. While this is effective in some cases and is reasonably benign, in many cases this adjuvant fails to induce a sufficiently protective response. It is also well known that antigens emulsified in a mineral oil vehicle together with whole mycobacterial cells (Freund's complete adjuvant, FCA) can produce a generally effective immune response against a

wide range of antigens. However, this formulation is unacceptable for routine use because of the inflammation, granulomas, ulceration and other lesions which can be formed at the injection site. Mineral oils alone (frequently referred to as Freund's Incomplete Adjuvant, FIA or Incomplete Freund's, ICF) are less damaging but are also less effective. Neutral oils (such as miglyol) and vegetable oils (such as arachis oil), ISCOMS and liposomes have also been used. Also effective are adjuvants containing purified mycobacterial component such as N-acetylmuramyl-L-alanyl-D-isogulutamine (MDP) or its analogues in aqueous or oil formulations. Among other adjuvants which have been or are currently used are the saponins, particularly triterpenoid mixtures such as Quil A (a purified extract from the bark of the tree *Quillaja saponarioa*) in aqueous solution or in the form of a matrix with cholesterol. Polycations such as diethylaminoethyldextran (DEAE dextran) can also be effective as adjuvants in some cases.

There have also been proposals to use a combination of two adjuvants substances in an adjuvant composition. For example, Australian patent no. 602348 describes an immunoadjuvant comprising an immunoadjuvant oil substantially free of mycobacteria and a polycationic polyelectrolyte immunoadjuvant such as DEAE dextran in the form of an emulsion having the polycationic polyelectrolyte dissolved in the aqueous phase. The two-component immunoadjuvant is said to overcome the rapid decline in the immune response associated with polycationic polyelectrolyte adjuvants on the one hand and, on the other, the weak initial response associated with immunoadjuvant oils. Accordingly, the two-component adjuvant is said to fill the gap in the prior art between those adjuvants inducing high peak/short life antibody responses and those inducing low peak/long life responses.

International application no. 88/07547 is primarily concerned with a novel peptide nevertheless, it also discloses the use of a novel adjuvant comprising DEAE

dextran and a saponin or aluminium hydroxide and notes an improved antibody titre when the two-component immunoadjuvants are used. In particular, solutions of DEAE dextran and saponin in phosphate buffered saline are used but there is no suggestion of the incorporation of an immunoadjavent oil into such compositions.

Australian patent no. 640414 discloses a solid vaccine composition comprising an antigenic substance capable of inducing the generation of antibodies on parenteral administration to an animal, a saponin and a polycationic adjuvant. The essence of the invention is that the vaccine is formulated as solid to be implanted in the animal to thereby induce a long-lasting immune response. There is no suggestion of the presence of an immunoadjuvants oil in the composition and, indeed, the specification teaches away from the use of an oil as it is critical to the invention that this formulation be solid.

In the present invention it has been found, surprisingly, that combinations of certain adjuvants enhance the effectiveness of an antigenic substance in stimulating an immune response to a much greater extent than the sum of the profiles that would be obtained by the use of the components separately or through the use of a two-component immunoadjuvant.

DISCLOSURE OF THE INVENTION

According to a first aspect of the present invention there is provided an adjuvant composition for stimulating an effective immune response in an animal to an antigenic substance when co-administered to said animal with said antigenic substance, comprising:

(a) a saponin with immune stimulating activity;

(b) a polycationic polyelectrolyte with immune stimulating activity; and

(c) an immunoadjuvant oil.

According to a second aspect of the present invention there is provided a vaccine for administration to an animal, comprising:

(1) an antigenic substance; and

(2) an adjuvant composition comprising:

(a) a saponin with immune stimulating activity;

(b) a polycationic polyelectrolyte with immune stimulating activity;

(c) an immunoadjuvant oil.

According to a third aspect of the present invention there is provided a method of stimulating an effective immune response in an animal to an antigenic substance, comprising the steps of:

(1) providing said antigenic substance;

(2) providing an adjuvant composition for stimulating an effective immune response to said antigenic substance, comprising:

(a) a saponin with immune stimulating activity;

(b) a polycationic polyelectrolyte with immune stimulating activity; and

(c) an immunoadjuvant oil; and

(3) challenging said animal with said antigenic substance and said adjuvant composition.

According to a fourth aspect of the present invention there is provided the use of an adjuvant composition comprising:

(a) a saponin with immune stimulating activity;

(b) a polycationic polyelectrolyte with immune stimulating activity; and

(c) an immunoadjuvant oil

to stimulate an effective immune response in an animal challenged with an antigenic substance.

According to a fifth aspect of the present invention there is provided the use of an adjuvant composition comprising:

(a) a saponin with immune stimulating activity;

(b) a polycationic polyelectrolyte with immune stimulating activity; and

(c) an immunoadjuvant oil

in the preparation of a medicament for administration to an animal, wherein said medicament further comprises an antigenic substance.

The saponins are common secondary constituents of plants and typically are glycosides composed of several (hydrophilic) sugars in association with a (hydrophobic) molecule, which can be either a steroid or triterpenoid structure. In particular, an extract from the South American tree *Quillaja saponarioa* shows good adjuvant activity and is now denoted "Quil A". While the precise chemical composition of Quil A is not known, the sugar moieties detected in the mixture include rhamnose, fucose, arabinose, xylose, galactose, glucose, apiose and glucuronic acid and the hydrophobic moiety has a triterpenoid structure. The nature of Quil A is discussed as length in Australian patent application no. 10777/95, the disclosure of which is incorporated herein by reference.

Preferably, the saponin is a triterpenoid compound or a mixture of triterpenoid compounds. More preferably, the saponin is Quil A or the extract disclosed in Australian application no. 10777/95, or compounds obtainable from these extracts. Still more preferably, the saponin is Quil A.

As used throughout the description and claims the term "polycationic polyelectrolyte" refers to polymer or oligomers, natural or synthetic, that, by virtue of their chemical structure, acquire a plurality of discrete positive charges in aqueous solution under appropriate pH conditions. Suitable polycationic polyelectrolytes are DEAE dextran, polyethyleneimine, ethoxylated polyethyleneimine, epichlorhydrin-modified polyethyleneimine, diethylaminoethyl ester and amide derivatives of acrylate polymers, copolymers and the like. The most preferable polycationic polyelectrolyte is DEAE dextran, which is a polycationic derivative of dextran (average molecular weight 10000 to 1000000, preferably 200000 to 750000, most preferably 500000) containing diethylamino ethyl groups linked to glucose in a 1:3 ratio. Typically the polycationic polyelectrolyte is in aqueous solution, for example, phosphate buffered saline.

While the invention embraces a wide range of immunoadjuvant oils, mineral oils are preferred. More preferred are those mineral oils already known in the art for use as adjuvants and including substances such as Drakeol, Markol, squalene, squalane and the like but the preferred mineral oil is Montanide oil. Mineral oil immunoadjuvants are frequently referred to as Freund's incomplete adjuvant and this adjuvant typically comprises 85% mineral oil and 15% mannide monooleate as an emulsifier.

Typically the adjuvant composition of the present invention takes the form of an emulsion with the polycationic polyelectrolyte dissolved in the aqueous phase and the mineral oil forming the non-aqueous phase. It is well known that immunoadjuvant emulsions of individual oils used separately can be formulated with oil to water phase ratios extending over a broad range and embracing the ratios 80:20 to 20:80 (v/v) for example, more preferably 60:40 to 40:60 (v/v). Such a broad range of ratios of oil phase to aqueous phase also applies in the present invention except that the aqueous phase will always comprise a polycationic polyelectrolyte solution and the composition will also include a saponin. While not wishing to be bound by theory, the saponin is amphiphilic and which may partition itself between the phases with the hydrophilic sugar residues in the aqueous phase and the hydrophobic triterpenoid structure in the non-aqueous phase. Accordingly, the saponin may serve to stabilise the emulsion.

Preferably, vaccines including adjuvant compositions in accordance with the present invention contain the saponin component at a concentration greater than 50µg/ml and the polycationic polyelectrolyte at a concentration of greater than 1mg/ml. More preferably, they contain saponins in a concentration of greater than 100µg/ml and the polycationic polyelectrolyte component in a concentration of greater than 1.5mg/ml. The upper limits of concentration of the saponin component and the

polycationic polyelectrolyte are essentially determined by economic considerations since these components are expensive, but the saponin may be present in concentrations up to 10mg/ml, typically up to 1mg/ml, and the polycationic polyelectrolyte may be present in concentrations up to 200mg/ml, typically 150mg/ml.

The emulsifiers used to form the novel compositions of the invention are those known in the art such as mannide monooleate, Arlacela A, Arlacela 80 and Tween 80. It will be recognised by those skilled in the art that the adjuvant composition can be used in virtually any vaccine including any antigenic substance, although it will be recognised that many factors other than the nature of the adjuvant composition will influence the nature of and level of the antibody response to the vaccine.

The adjuvant composition is particularly useful when used in conjunction with a whole cell killed vaccine or killed viral vaccine or a vaccine comprising a proteinaceous substance, which may or may not be glyocosylated or otherwise chemically modified, alone or as a carrier for a low molecular weight compound. In general, the antigenic substance will give rise to an immune response against a disease-causing agent but may also give rise to antibodies against an agent (such as a hormone) which does not normally give rise to a disease. The disease causing agent may be a structural component or toxin of a virus, bacteria or other microbe. Examples of virally-caused diseases which may be controlled by vaccines including the adjuvant composition of the present invention include infectious bursal disease virus, Newcastle disease, infectious bronchitis virus, pseudorabies, parvovirus, classical swine fever, equine influenza, bovine viral diarrhoea virus and canine corona virus. Examples of bacterially-caused diseases include atrophic rhinitis, loptospirosis, clostridial infections, bordetella brochisepticum infections in cats, coryza in poultry, fowl chloera, Mycoplasma gallisepticum infections in poultry, pleuropmeumonia and rabies. The adjuvant composition may

also be used in conjunction with sub-unit vaccines produced using recombinant DNA technology such as in a sub-unit vaccine against cattle ticks.

The antigenic substance may also comprise a target low molecular weight compound conjugated to a carrier selected so as not to be recognised by the organism as "self" and thereby to generate an immune response against the low molecular weight compound. Suitable carriers include fetuin, ovalbumin, bovine serum albumin, foetal calf serum and human serum albumin. Alternatively, the carrier may be keyhole limpet haemocyanin or beta-galactosidase, among others. The low molecular weight compound may be conjugated to the carrier by any convenient means. Suitable conjugators include glutaraldehyde, toluene diisocyanate, carbodiimide, or any other suitable conjugator.

The small molecules which may be conjugated to a character include toxins such as phomopsin or other substances such as mammaliam hormones or steroids against which it may be desirable to raise an immune response.

Other antigens which may be employed include red blood cells and virus like particles, particularly VLP/NS2.

Preferably, the antigenic substance is a fetuin-phomopsin conjugate, phomopsin A conjugated to ovalbumin, phomopsin A-fetal calf serum conjugate, a virus-like particle, particularly VLP/NS2 (a VLP comprising a blue tongue virus antigen encoded by a recombinant baculovirus vector), sheep red blood cells, or ovalbumin.

In the method of the invention the antigenic substance and adjuvant composition are conveniently mixed prior to administration. Typically, the antigenic substance is in aqueous solution, such as phosphate-buffered saline. The polycationic polyelectrolyte may also be in aqueous solution, such as in solution in phosphate-buffered saline, and together these components form the aqueous phase of the emulsion. However, it will be appreciated that the antigenic substance and adjuvant may be administered sequentially, and even that the various components of the

adjuvant composition may be administered sequentially rather than simultaneously provided that they undergo a physiological interaction *in vivo*.

Throughout the specification, except where the context requires otherwise due to express language or necessary implication, the word "comprising" is used in the sense of "including", ie. the features specified may be associated with further features in various embodiments of the invention and are not to be construed, necessarily, as the only features of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will now be further described, by way of example only, with reference to the accompanying drawings, in which:

Figure 1 shows the antibody response to sheep red blood cells in chickens in a Haemagglutination assay;

Figure 2 shows the antibody response to sheep red blood cells in chickens in an ELISA assay on 0.1ml of 0.1% sheep red blood cells/well coated plate;

Figure 3 shows the antibody response to phomopsins in cattle in an ELISA assay on 50 ng phomopsins/0.1ml/well coated plate;

Figure 4 shows the antibody response to ovalbumin in cattle in an ELISA assay on 50 ng ovalbumin/0.1ml/well coated plate;

Figure 5 shows the antibody response to phomopsins in wethers in an ELISA assay on 50ng phomopsins/0.1ml/well coated plate;

Figure 6 shows the antibody response to foetal calf serum in wethers in an ELISA assay on 50ng foetal calf serum/0.1ml/well coated plate; and

Figure 7 shows the antibody response to ovalbumin in Long-Evans hooded rats using an ELISA assay on 50 ng ovalbumins/0.1ml/well coated plate.

MODES OF CARRYING OUT THE INVENTION

Example 1

In this example a fetuin-phomopsin conjugate was employed as the antigen in a comparison of three adjuvant

formulations including one formulation conforming to the invention described in this specification. Sheep (12 per group) were the animal species used. The dose volume (1ml) and antigen concentration were kept constant and the oil component was 85% (v/v) mineral oil and 15% (v/v) mannide monooleate (incomplete Freund's adjuvant, ICF) in all formulations.

In one formulation the adjuvant comprised a 10mg/dose of DEAE dextran as the cationic polymer in incomplete Freund's oil (10mg DEAE/ICF). In another the adjuvant was 0.5mg Quil A as a triterpenoid component in incomplete Freund's oil (0.5mg Quil A/ICF) and in the third, representative of this invention, 5mg of DEAE and 0.5mg of Quil A were mixed together in incomplete Freund's oil (0.5mg Quil A/5mg DEAE/ICF). A primary and one booster injection were administered 16 weeks apart. Anti-phomopsin antibody titres (measured by ELISA) were not detectable prior to vaccination. Table 1 shows anti-phomopsin antibody titres 2 and 8 weeks after the booster injection.

TABLE 1

Adjuvant formulation	Dose volume	Sheep Nos.	Titre - Booster plus 2 weeks	Titre - Booster plus 8 weeks
10mg DEAE/ ICF	1ml	12	55,000	8,000
0.5mg Quil A/ ICF	1ml	12	23,000	2,000
0.5mg Quil A/ 5mg DEAE/ICF	1ml	12	99,000	37,000

The results show that the trivalent adjuvant prepared according to this invention gave a significantly higher antibody titre two weeks after the booster injection than either of the divalent formulations lacking one component of the trivalent formulation. The titre obtained with the trivalent adjuvant was also greater than the sum of the titres produced by the two divalent formulations demonstrating an unexpected synergism between the components. Eight weeks after the booster injection the trivalent vaccine was out-performing the divalent formulations by an increased margin demonstrating the

longer duration of effect and the synergistic response achieved.

Example 2

In another comparison of two, two-component mixtures and a three component mixture representative of this invention, three groups of 12 sheep were injected with a fetuin-phomopsin conjugate antigen formulated in an adjuvant mixture of DEAE dextran in incomplete Freund's adjuvant (ICF) or Quil A in ICF or a mixture of DEAE dextran and Quil A in ICF. The antibody titres achieved are shown in Table 2.

TABLE 2

Adjuvant formulation	Dose volume	Sheep Nos.	Titre - Booster plus 2 weeks	Titre - Booster plus 8 weeks
10mg DEAE/ ICF	1ml	12	8,000	2,000
0.5mg Quil A/ ICF	1ml	12	9,000	1,000
0.5mg Quil A/ 5mg DEAE/ICF	1ml	12	44,000	14,000

Once again the adjuvant formulated according to this invention demonstrates an enhanced, synergistic, long-lasting effect when compared to two component formulations in which one of the three components specified in this invention is missing.

Example 3

In this example a comparison was made between two trivalent adjuvant formulations incorporating a triterpinoid (Quil A), a cationic polymer (DEAE dextran) and two different commercially available oils (ICF or Montanide 888). Both formulations incorporated a commercial preservative, Thimerosal, and the antigen was a phomopsin fetuin conjugate. The results are shown in Table 3

TABLE 3

Adjuvant formulation	Dose volume	Sheep Nos.	Anti-phomopsin titre - Booster plus 2 weeks	Anti-phomopsin titre - Booster plus 8 weeks
0.5mg Quil A/5mg DEAE/ ICF	1ml	11	20,000	16,000
0.5mg Quil A/5mg DEAE/ Montanide 888	1ml	11	40,000	25,000

The results demonstrate the high titres obtained with this invention and the longevity of the effect with both formulations. A better response is seen with the Montanide oil under the conditions used demonstrating that careful selection of the oil component of the invention from a number of available products can give advantage for particular applications.

Example 4

Animal species: Rabbit

The antigen: Virus-like particles (VLPs).

Adjuvant formulations:

This invention. 2.5mg Quil A and 50mg DEAE-dextran in 3ml of PBSA was filtered through a 0.2µm filter. Six hundred microlitres of this solution was added to 200 microlitres of antigen (1mg VLP/NS2, a VLP comprising a blue tongue virus antigen encoded by a recombinant baculovirus vector, pelleted and resuspended in 200 microlitres PBSA). 1.2 ml of Montanide ISA 50V was then added to this combined solution. The mixture was sonicated and emulsified to form a viscous liquid.

Freund's complete. 1ml Freund's Complete adjuvant was added to 1 ml VLP/NS2 (1mg) in PBSA. This solution was sonicated and emulsified. An extremely viscous, almost solid emulsion was formed.

Freund's incomplete/DEAE dextran. 1 mg VLP/NS2 was resuspended in 1ml of 15% DEAE-dextran and added to 1ml of Freund's incomplete adjuvant. This solution was sonicated and emulsified.

PBSA. VLP/NS2 was dissolved in PBSA at a concentration of 1mg per ml.

Vaccination protocols

This invention. Five 0.1ml intradermal injections per rabbit were given for the primary vaccination and 0.3 ml in each hind leg were given for the booster.

Freund's complete. Four intradermal injections of 0.1ml were given as the primary vaccination and 0.4ml intramuscular injection per hind leg was given as a booster.

Freund's incomplete/DEAE dextran. Five intradermal injections were given per rabbit were given as the primary vaccination and 0.3ml per hind leg were given as a booster.

PBSA. A 0.5ml intramuscular injection was given to each hind legs for both the primary and booster injections.

The rabbits were bled on day 1 and injections were given on day 5, day 54, and day 78.

This example demonstrates that the adjuvant composition of the present invention performs very well when compared to Freund's adjuvant. In the data shown in Table 4 it will be apparent that the immune response using the adjuvant of the present invention begins earlier than the immune response when Freund's adjuvant is used and is stronger and more long lasting. In addition, the adjuvant does not induce the formation of lesions at the injection site as Freund's adjuvant can.

Results.

The antibody titres achieved for each protocol are given in Table 4.

TABLE 4

Rabbit	Day 1	Day 20	Day 44	Day 61	Day 72	Day 89	Day 96	Day 109	Day 123
This invention	0	1000	5000	>50000	>50000	>50000	>50000	>50000	25000
This invention	0	25000	25000	>50000	>50000	>50000	25000	5000	1000
Freund's	0	0	5000	5000	25000	>50000	25000	25000	1000
Freund's	0	0	1000	1000	5000	25000	>50000	5000	5000
ICF	0	0	5000	25000	50000	25000	5000	5000	1000
ICF	0	1000	5000	5000	5000	>50000	25000	25000	5000
PBS	0	0	1000	5000	1000	1000	5000	1000	1000
PBS	0	0	1000	50000	5000	5000	5000	1000	0

The results demonstrate the effectiveness of an adjuvant encompassed by this invention compared to Freund's complete adjuvant, ICF and the antigen injected in PBSA as a control.

Example 5

In this example a comparison was made between the immune response and injection site reaction of chickens to sheep red blood cells. The red blood cells were administered either in Freund's complete adjuvant with a booster injection, in incomplete Freund's adjuvant or in an adjuvant system typifying this invention for both primary and booster injections.

Sterile sheep blood (100 ml) was collected and 1 volume of blood was added immediately into 1.2 volume of Alsever's solution (Methods in Immunology and Immunochemistry, vol 4, 41, Eds: Williams, C.A. and Chase, M.W., 1977).

Hybrid white leghorn chickens eggs (Ex SPF Unit) were set on 2/10/97 and chickens were hatched 21 days later.

Five week old chickens were weighed and divided into two groups of 12. 0.5 to 1ml blood was collected from the wing vein of each chicken prior to vaccination. For the primary injections formulated using an adjuvant typifying that described in this invention, 0.1 ml of sheep red blood cells were added to 0.1 ml of phosphate buffered saline containing 62.5 µg Quil A, 1.25 mg DEAE-dextran and emulsified with 0.3 ml of Montanide 888 oil (60 %). In the comparison group, 0.1 ml of sheep red blood cells were added to 0.15 ml of phosphate buffered saline and emulsified with 0.25 ml of Complete Freund's adjuvant (50 %). In both groups, the total volume injected was 0.5 ml per dose. It was administered in equal volumes to the thigh muscles of both legs.

After two weeks the chickens were weighed, tissue reactions at the injection sites were inspected and 0.5 to 1 ml blood was collected from wing vein.

After a further 13 days the chickens were weighed once again, tissue reactions at the injection sites were inspected and 0.5 to 1 ml blood was collected from wing vein. After the inspection a booster injection of 0.1 ml of sheep red blood cells was given intra muscularly in both adjuvants as for the primary injection but incomplete

Freund's adjuvant was employed in the comparison group.

Two weeks later the chickens were weighed, tissue reactions at the injection sites were inspected and 0.5 to 1 ml blood was collected from wing vein.

Eight weeks after the booster injection the chickens were weighed, tissue reactions at the injection sites were inspected and 0.5 to 1 ml blood was collected from wing vein.

Haemagglutination Assay

Chickens sera were incubated at 56°C for 30 minutes to inactivate complement. Fifty µl of phosphate buffered saline was added to all wells of row 1 to 12 of 96 wells, U-shaped bottom, microtest plates (Sarstedt, Australia). Fifty µl of heat inactivated sera, before and after the immunisation, were added to wells of the first row. Two fold serial dilutions were performed across the plates. Fifty µl of 2 % sheep red blood cells suspension was added to all wells. The plates were shaken for 1 minute, covered and incubated at 4°C for 2 hours. Titres were expressed as the reciprocal of the highest dilution resulting in complete agglutination.

ELISA on sheep red blood cells coated microtitre plates

Sheep red blood cells were diluted as 0.1 % in carbonate coating buffer pH 9.6 and 100 µl of the solution was added to all wells of row 2 to 12 of 96 wells, flat bottom, microtitre plates (Nunc-Immuno plate, F 96 polysorp, Cat. 475094). After overnight incubation at 4°C, the plates were washed four times with 0.05 % tween 20 in saline. After the washing, 100 µl of 0.1 % gelatine in phosphate buffered saline was added to all wells of row 2 to 12 of microtitre plates. Which was followed by the addition of the reference serum and sera for testing, diluted 1/100 in 0.1 % gelatine in phosphate buffered saline, to the wells of row 2. Two fold serial dilutions were performed across the plates. After 2 hours incubation at room temperature, the plates were washed four times and 100 µl of 1/20,000 anti-chicken IgG, developed in rabbit,

conjugated to peroxidase (Sigma Cat. A 9046) was added and incubated for a further 1 hour. After washing the plate four times 3, 3', 5, 5'- tetramethylbenzidine (Sigma Cat. T2885) substrate was added and incubated for a further 15 minutes before the stopping solution was added. Titres were expressed as the reciprocal of the dilution resulting in 0.5 optical density of the wells.

The results of the experiment are shown in Figure 1 and 2 and Tables 5 to 7.

Table 5. Adjuvant experiment in chickens (hybrid white leghorn)

Adjuvant	Isolator	Pink No.	Sex	Weight (gm)					Tissue reaction 2 weeks after prime	Tissue reaction 2 weeks after boost 1	Remark 8 weeks after boost 1
				Before prime	2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1			
(Quil A/ DEAE/ 888 oil)	4	41	Female	310	500	680	909	1372			
	4	42	Female	315	529	722	940	1389			
	4	43	Female	285	464	617	845	1298			
	4	44	Male	336	584	794	1156	1875			
	4	45	Male	433	715	955	1322	2137			
	4	46	Male	345	586	738	1084	1663			
	3	47	Female	287	475	645	841	977			Head pecked by others
	3	48	Female	361	590	767	941	1290			
	3	49	Female	309	408	646	831	1162			
	3	50	Male	398	651	895	1159	1585			
	3	51	Male	390	675	946	1307	1832			
	3	52	Male	439	726	1047	1303	1980			
Mean				351	575	786	1053	1548			
SD				54	103	143	192	356			
Counts				12	12	12	12	12			
SE				16	30	41	55	103			

Adjuvant	Isolator	Yellow No.	Sex	Weight (gm)					Tissue reaction 2 weeks after prime	Tissue reaction 2 weeks after boost 1	Tissue reaction 8 weeks after boost 1
				Before prime	2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1			
CF/ICF	4	1	Female	313	527	700	953	1435			
	4	2	Female	310	482	610	843	1184			
	4	3	Female	308	511	680	940	1318			
	4	4	Male	354	636	834	1185	1904	Lump on left leg	Lump on left leg	
	4	5	Male	323	573	755	1092	1884			
	4	6	Male	388	664	830	1160	2006			
	3	7	Female	317	518	680	902	970			Head pecked by others
	3	8	Female	315	518	736	956	1397			
	3	9	Female	348	557	752	986	1416			
	3	10	Male	415	706	1002	1325	1965			
	3	11	Male	420	724	1033	1358	1957			
	3	12	Male	279	463	714	931	1458			Head pecked by others
Mean				339	573	777	1052	1575			
SD				44	88	128	169	352			
Counts				12	12	12	12	12			
SE				13	25	37	49	102			

Table 6. Adjuvant experiment in chickens (hybrid white leghorn)

Adjuvant	Isolator	Pink no.	Sex	Titre using haemagglutination assay (reciprocal dilution)			
				2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	4	41	Female	32	4	16	8
	4	42	Female	16	8	64	8
	4	43	Female	8	8	64	16
	4	44	Male	32	8	32	4
	4	45	Male	16	4	16	4
	4	46	Male	16	8	16	4
	3	47	Female	64	16	64	2
	3	48	Female	64	32	128	16
	3	49	Female	8	8	64	8
	3	50	Male	64	8	32	4
	3	51	Male	16	8	32	4
	3	52	Male	64	16	128	16
Mean				33	11	55	8
SD				24	8	39	5
Counts				12	12	12	12
SE				7	2	11	2

Adjuvant	Isolator	Yellow no.	Sex	Titre using haemagglutination assay (reciprocal dilution)			
				2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
CF/ICF	4	1	Female	4	4	8	8
	4	2	Female	8	2	4	4
	4	3	Female	4	2	8	4
	4	4	Male	4	8	32	32
	4	5	Male	16	16	16	4
	4	6	Male	4	4	8	4
	3	7	Female	16	8	32	4
	3	8	Female	16	4	16	32
	3	9	Female	16	16	64	64
	3	10	Male	4	32	32	8
	3	11	Male	8	8	8	4
	3	12	Male	2	2	16	8
Mean				9	9	20	15
SD				6	9	17	19
Counts				12	12	12	12
SE				2	3	5	5

Table 7. Adjuvant experiment in chickens (hybrid white leghorn)

Adjuvant	Isolator	Pink no.	Sex	Titre using ELISA (x 1000)			
				2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	4	41	Female	35	12	85	11
	4	42	Female	22	9	115	14
	4	43	Female	36	20	43	21
	4	44	Male	36	18	189	12
	4	45	Male	19	13	42	15
	4	46	Male	106	35	431	17
	3	47	Female	54	24	250	21
	3	48	Female	36	15	82	33
	3	49	Female	5	4	34	10
	3	50	Male	34	42	191	27
	3	51	Male	20	14	42	10
	3	52	Male	60	32	191	20
Mean				38	20	141	18
SD				26	11	117	7
Counts				12	12	12	12
SE				8	3	34	2

Adjuvant	Isolator	Yellow no.	Sex	Titre using ELISA (x 1000)			
				2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
CF/ICF	4	1	Female	1	3	9	4
	4	2	Female	2	2	5	6
	4	3	Female	2	2	7	8
	4	4	Male	1	5	17	10
	4	5	Male	6	19	22	5
	4	6	Male	3	4	12	8
	3	7	Female	2	3	12	6
	3	8	Female	2	4	5	6
	3	9	Female	2	9	15	14
	3	10	Male	1	15	29	6
	3	11	Male	2	9	13	8
	3	12	Male	0	1	4	3
Mean				2	6	12	7
SD				1	6	7	3
Counts				12	12	12	12
SE				0	2	2	1

Example 6

In this example a comparison was made between cattle injected with phomopsin A conjugated to ovalbumin in Freund's complete adjuvant with a booster injection of the conjugate antigen in incomplete Freund's adjuvant or with the same antigen delivered in an adjuvant prepared according to this invention.

Twenty four, five months old cattle were weighed and two 10 ml samples of blood were collected from each animal. All the cattle also received 4 ml of five in one vaccine, injected subcutaneously to the left side of the back of the neck.

Two weeks later the cattle were weighed and divided into two randomised groups. The animals were then given a primary injection. The animals in one group received an injection of 100 µg phomopsin A conjugated to 336 µg ovalbumin, 1 mg Quil A and 10 mg DEAE-dextran dissolved in 0.8 ml of sterile water and emulsified with 1.2 ml of Montanide 888 oil (60 %). The animals in the other group were injected with 100 µg phomopsin A conjugated to 336 µg ovalbumin dissolved in 1 ml of sterile water, and emulsified with 1 ml of Complete Freund's adjuvant (50 %). In both groups, immunogens were injected subcutaneously as a total volume of 2 ml to the right side of the back of the neck, below the ear.

Thirteen days later tissue reactions at the injection sites were inspected and 10 ml blood was collected from jugular vein.

After four weeks cattle were weighed, tissue reactions at the injection sites were inspected and 10 ml blood was collected from jugular vein. A booster injection of 100 µg phomopsin A conjugated to 440 µg foetal calf serum was given subcutaneously as in primary injection. In the case of the comparison group this was formulated in Incomplete Freund's Adjuvant. A second dose of five in one vaccine was also injected subcutaneously to the left side of the back of the neck.

Two weeks later tissue reactions at the injection sites were inspected and 10 ml blood was collected from jugular vein.

Eight weeks after the booster injection cattle were weighed and tissue reactions at the injection sites were inspected and 10 ml blood was collected from jugular vein.

ELISA on 50 ng phomopsins/well coated microtitre plates

Phomopsins were diluted as 50 ng/100 µl in carbonate coating buffer pH 9.6 and 100 µl of the solution was added to all wells of row 2 to 12 of 96 wells, flat bottom, microtitre plates (Sarstedt, Australia). After overnight incubation at 40°C, the plates were washed four times with 0.05 % tween 20 in saline. After the washing, 100 µl of 0.1 % gelatine in phosphate buffered saline was added to all wells of microtiter plates. Reference serum and sera for testing, diluted 1/100 in 0.1 % gelatine in phosphate buffered saline, were then added to the wells of row 2. Two fold serial dilutions were performed across the plates. After 2 hours incubation at room temperature, the plates were washed four times and 100 µl of 1/15,000 anti-bovine IgG, developed in rabbit, conjugated to peroxidase (Sigma Cat.B 1520) was added and incubated for a further 2 hour. After washing the plate four times 3, 3', 5, 5'-tetramethylbenzidine (Sigma Cat. T2885) substrate was added and incubated for a further 20 minutes before the stopping solution was added. Titres were expressed as the reciprocal of the dilution resulting in 0.5 optical density of the wells.

ELISA on 50 ng ovalbumin/well coated microtitre plates

ELISA was also performed on 50 ng ovalbumin/well coated plates as above in phomopsins 50 ng/well coated plates except 96 wells, flat bottom, Nunc-Immuno maxisorp microtitre plates (Cat. 439454) were used for the assay.

The results obtained are shown in Figures 3 and 4 and Tables 8 to 12.

Table 8. Adjuvant trial in cattle

Adjuvant	Animal no. Left	Sex	Animal no. Right	Weight (kg)				
					Before prime	Before boost 1	Before boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	5	Female	373	148	164	186	210	210
	6	Male	9721	183	203	240	282	282
	14	Male	9701	173	188	226	256	256
	15	Male	9713	153	171	194	230	230
	20	Female	998	138	152	169	194	194
	24	Female	285	160	174	194	221	221
	25	Male	342	135	152	182	202	202
	28	Female	9723	184	200	227	256	256
	30	Female	9710	160	180	209	224	224
	31	Male	9732	157	176	209	230	230
	34	Male	631	155	164	177	210	210
	35	Female	9720	165	178	183	216	216
	37	Female	425	176	186	211	241	241
Mean				160	176	201	229	229
Counts				13	13	13	13	13
SD				15	16	22	25	25
SE				4	4	6	7	7

Adjuvant	Animal no. Left	Sex	Animal no. Right	Weight (kg)				
					Before prime	Before boost 1		8 weeks after boost 1
CF/ICF	4	Male	9726	143	148	162	199	199
	8	Female	9709	140	150	167	185	185
	10	Female	9705	155	168	185	205	205
	13	Male	979	157	175	187	215	215
	17	Male	983	183	204	221	252	252
	19	Female	215	168	193	220	255	255
	22	Female	377	167	173	169	200	200
	23	Female	263	146	160	162	206	206
	26	Male	9730	176	188	214	232	232
	32	Female	466	160	178	199	222	222
	36	Male	418	116	124	143	174	174
Mean				155	169	184	213	213
Counts				11	11	11	11	11
SD				19	23	26	26	26
SE				6	7	8	8	8

Table 9. Adjuvant trial in cattle

Adjuvant	Animal no. Left	Sex	Animal no. Right	Tissue reactions to phomopsins-ovalbumin conjugate (2 ml/dose)			
				2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	5	Female	373				
	6	Male	9721			3x3 cm	
	14	Male	9701				
	15	Male	9713				
	20	Female	998				
	24	Female	285				
	25	Male	342				
	28	Female	9723			3x5 cm	3x4 cm
	30	Female	9710				
	31	Male	9732	7x5 cm	7x5 cm	4x5 cm	3x3 cm
	34	Male	631			3x3 cm	
	35	Female	9720				
	37	Female	425				

Adjuvant	Animal no. Left	Sex	Animal no. Right	Tissue reactions to phomopsins-ovalbumin conjugate (2 ml/dose)			
				2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
CF/ICF	4	Male	9726				3x3 cm
	8	Female	9709	8x5 cm		6x4 cm	8x6 cm
	10	Female	9705	10x3 cm	6x5 cm		3x4 cm
	13	Male	979				
	17	Male	983	12x6 cm	8x6 cm	2x2 cm	
	19	Female	215	8x5 cm	4x8 cm	4x5 cm	
	22	Female	377				
	23	Female	263			6x7 cm	3x3 cm
	26	Male	9730		7x5 cm		
	32	Female	488			8x8 cm	4x5 cm
	36	Male	418	10x6 cm		7x8 cm	7x6 cm

Table 10. Adjuvant trial in cattle

Adjuvant	Animal no. Left	Sex	Animal no. Right	Tissue reactions to 5 in 1 vaccine (4 ml/dose)			
				4 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	5	Female	373				
	6	Male	9721				
	14	Male	9701				
	15	Male	9713		2x2 cm	2x3 cm	3x5 cm
	20	Female	998	4x3 cm	4x4 cm	1x1 cm	
	24	Female	285				
	25	Male	342		2x2 cm	8x5 cm	5x5 cm
	28	Female	9723				
	30	Female	9710			5x4 cm	5x6 cm
	31	Male	9732			5x5 cm	3x4 cm
	34	Male	631		4x4 cm	3x3 cm	
	35	Female	9720	3x2 cm	3x2 cm	7x5 cm	
	37	Female	425	4x2 cm	3x3 cm		

Adjuvant	Animal no. Left	Sex	Animal no. Right	Tissue reactions to 5 in 1 vaccine (4 ml/dose)			
				4 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
CF/ICF	4	Male	9726	2x2 cm		6x3 cm	3x3 cm
	8	Female	9709		2x2 cm	1x1 cm	
	10	Female	9705			6x4 cm	3x5 cm
	13	Male	979			7x8 cm	
	17	Male	983	5x3 cm	5x5 cm	3x4+10x10 cm	
	19	Female	215				
	22	Female	377			3x3 cm	
	23	Female	263	5x4 cm		3x3 cm	
	26	Male	9730	3x2 cm		3x4 cm	3x3 cm
	32	Female	466				
	36	Male	418			3x5 cm	

Table 11. Adjuvant trial in cattle

Adjuvant	Animal No.	Sex	Anti-phomopsin IgG titre (x100)			
			2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	5	Female	16	38	120	95
	6	Male	7	17	134	60
	14	Male	26	170	514	158
	15	Male	12	20	99	51
	20	Female	4	22	119	74
	24	Female	17	121	286	92
	25	Male	11	17	50	24
	28	Female	26	162	340	116
	30	Female	16	33	86	46
	31	Male	29	81	261	156
	34	Male	5	23	94	59
	35	Female	14	74	200	191
	37	Female	8	50	237	231
Mean			15	64	195	104
Counts			13	13	13	13
SD			8	55	131	63
SE			2	15	36	17

Adjuvant	Animal No.	Sex	Anti-phomopsin IgG titre (x100)			
			2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
CF/ICF	4	Male	7	17	60	129
	8	Female	12	64	291	299
	10	Female	2	27	186	173
	13	Male	10	45	186	93
	17	Male	8	74	299	142
	19	Female	5	19	162	28
	22	Female	11	104	595	128
	23	Female	4	45	205	128
	26	Male	9	41	256	302
	32	Female	10	70	231	240
	36	Male	3	19	94	82
Mean			7	48	233	158
Counts			11	11	11	11
SD			3	28	141	88
SE			1	8	43	27

Table 12. Adjuvant trial in cattle

Adjuvant	Animal No.	Sex	Anti-ovalbumin IgG titre (x100)			
			2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	5	Female	4	5	8	15
	6	Male	6	8	78	43
	14	Male	2	40	110	63
	15	Male	2	3	13	28
	20	Female	2	9	77	36
	24	Female	3	9	78	22
	25	Male	2	3	37	35
	28	Female	1	19	35	43
	30	Female	5	5	32	24
	31	Male	2	5	29	15
	34	Male	1	2	9	6
	35	Female	0	4	25	51
	37	Female	2	4	49	19
Mean			2	9	45	31
Counts			13	13	13	13
SD			2	10	32	16
SE			0	3	9	4

Adjuvant	Animal No.	Sex	Anti-ovalbumin IgG titre (x100)			
			2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
CF/ICF	4	Male	2	3	10	29
	8	Female	2	23	84	113
	10	Female	1	6	19	16
	13	Male	2	4	60	37
	17	Male	4	15	147	181
	19	Female	2	5	51	34
	22	Female	3	15	63	73
	23	Female	1	15	85	237
	26	Male	2	6	52	173
	32	Female	5	42	178	285
	36	Male	2	8	70	77
Mean			3	13	75	114
Counts			11	11	11	11
SD			1	12	50	92
SE			0	3	15	28

Example 7

In this example a comparison was made between sheep injected with a phomopsin A- fetal calf serum conjugate in Freund's complete adjuvant with a booster injection given in incomplete Freund's adjuvant and the same antigen injected in an adjuvant formulation prepared according to this invention.

Primary injections were started during marking of 10 to 12 weeks old lambs. Twenty four, ten to twelve weeks old lambs, weighing between 10 to 20 kg, were weighed and 10 ml blood was collected from jugular vein. They were divided into two equal groups randomised according to weight. One group was injected with 50 µg phomopsin A conjugated to 220 µg foetal calf serum, 0.5 mg Quil A and 5 mg DEAE-dextran dissolved in 0.8 ml of sterile water and emulsified with 1.2 ml of Montanide 888 oil (60 %). The second group was injected with phomopsin A 50 µg conjugated to foetal calf serum 220 µg dissolved in 1 ml of sterile water, and emulsified with 1 ml of Complete Freund's adjuvant. Both groups were injected subcutaneously with 2 ml to the right side of the back of the neck, below the ear. In addition all lambs received an injection of 2 ml of six in one plus selenium vaccine, injected subcutaneously at a separate site.

Two weeks after the primary injection lambs were weighed, tissue reactions at the injection sites were inspected and 10 ml blood was collected from jugular vein. A second dose of six in one plus selenium vaccine was injected subcutaneously at the back of the neck behind the ear.

Three months after the primary injection lambs were weighed, tissue reactions at the injection sites were inspected and 10 ml blood was collected from jugular vein. A booster injection of phomopsin A 50 µg conjugated to 220 µg foetal calf serum was given subcutaneously as in primary injection. In the case of the comparison group Incomplete Fruend's Adjuvant was used as the adjuvant in place of

Freund's complete adjuvant.

Two weeks after the booster injection lambs were weighed, tissue reactions at the injection sites were inspected and 10 ml blood was collected from jugular vein.

Eight weeks after the booster injection lambs were weighed and tissue reactions at the injection sites were inspected and 10 ml blood was collected from jugular vein.

ELISA on 50 ng phomopsins/well coated microtitre plates

Phomopsins were diluted as 50 ng/100 µl in carbonate coating buffer pH 9.6 and 100 µl of the solution was added to all wells of row 2 to 12 of 96 wells, flat bottom, microtitre plates (sarstedt, Australia). After overnight incubation at 40°C, the plates were washed four times with 0.05 % tween 20 in saline. After the washing, 100 µl of 0.1 % gelatine in phosphate buffered saline was added to all wells of microtiter plates. Reference serum and sera for testing, diluted 1/100 in 0.1 % gelatine in phosphate buffered saline, were then added to the wells of row 2. Two fold serial dilutions were performed across the plates. After 2 hours incubation at room temperature, the plates were washed four times and 100 µl of 1/14,000 anti-sheep IgG, developed in donkey, conjugated to peroxidase (Sigma Cat. A 3415) was added and incubated for a further 2 hour. After washing the plate four times 3, 3', 5, 5'-tetramethylbenzidine (Sigma Cat. T2885) substrate was added and incubated for a further 20 minutes before the stopping solution was added. Titres were expressed as the reciprocal of the dilution resulting in 0.5 optical density of the wells.

ELISA on 50 ng foetal calf serum/well coated microtitre plates

ELISA was also performed on 50 ng foetal calf serum/well coated plates as above in phomopsins 50 ng/well coated plates except 96 wells, flat bottom, Nunc-Immuno polysorp microtitre plates (Cat. 475094) were used and coating was done at 4°C.

The results obtained are shown in Figures 5 and 6 and

Tables 13 to 15.

Table 13. Adjuvant trial in wethers

Adjuvant	Animal no.	Weight (kg)				
		Before prime	2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	85	11.5	18.7	28.6	31.0	28.0
	86	14.0	18.3	28.4	30.0	26.5
	87	15.5	20.2	20.2	19.0	15.5
	88	14.5	21.4	32.4	35.5	29.5
	89	14.5	19.1	32.0	33.5	30.5
	90	16.5	22.6	23.8	33.5	29.5
	91	15.0	20.2	31.0	30.0	24.0
	92	11.5	16.1	24.0	26.5	23.0
	93	16.0	21.6	30.6	33.0	30.0
	94	12.5	19.6	28.2	31.0	26.5
	95	13.0	19.5	26.4	28.0	23.5
	96	13.5	20.2	34.2	37.5	34.5
Mean		14.0	19.8	28.3	30.7	26.8
SD		1.65	1.70	4.11	4.80	4.86
Counts		12	12	12	12	12
SE		0.48	0.49	1.19	1.39	1.40

Adjuvant	Animal no. Left	Weight (kg)				
		Before prime	2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
CF/ICF	145	15.0	20.4	29.8	34.0	31.5
	146	13.0	18.2	28.6	32.5	29.5
	147	10.5	13.5	15.6	17.0	12.5
	148	12.5	16.3	27.2	30.0	27.0
	149	16.0	21.4	32.4	35.5	30.5
	150	17.5	22.0	36.2	36.0	31.0
	151	13.5	17.3	26.2	28.0	24.5
	152	18.0	24.2	37.2	38.5	34.5
	153	17.5	22.4	31.2	30.5	28.0
	154	16.0	20.4	27.4	30.0	25.0
	155	15.5	19.5	32.2	35.0	31.0
	156	14.0	19.7	25.2	27.5	25.0
Mean		14.9	19.6	29.1	31.2	27.5
SD		2.28	2.93	5.67	5.62	5.64
Counts		12	12	12	12	12
SE		0.66	0.85	1.64	1.62	1.63

By eight weeks after boost 1 all sheep lost body weight due to the drought and lack of food

Table 14. Adjuvant trial in wethers

Adjuvant	Animal No.	Anti-phomopsin IgG titre (x1000)			
		2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	85	21	5	95	57
	86	14	22	281	145
	87	26	7	68	33
	88	20	10	59	72
	89	50	215	328	565
	90	31	15	165	71
	91	37	40	662	640
	92	28	9	75	56
	93	19	10	92	54
	94	8	27	61	86
	95	15	8	84	56
	96	7	24	98	62
Mean		23	33	172	158
Counts		12	12	12	12
SD		12	58	178	210
SE		4	17	51	61

Adjuvant	Animal No.	Anti-phomopsin IgG titre (x1000)			
		2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
CF/ICF	145	9	49	211	62
	146	11	50	120	51
	147	21	144	274	219
	148	14	51	105	83
	149	22	219	256	140
	150	45	203	298	351
	151	15	48	121	80
	152	36	107	365	494
	153	22	56	151	191
	154	20	188	197	165
	155	22	49	252	109
	156	11	134	173	252
Mean		21	108	210	183
Counts		12	12	12	12
SD		11	67	81	132
SE		3	19	23	38

Table 15. Adjuvant trial in wethers

Adjuvant	Animal No.	Anti-foetal calf serum IgG titre (x100)			
		2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	85	6	7	120	127
	86	17	41	403	176
	87	20	15	148	53
	88	13	25	132	123
	89	71	238	656	343
	90	14	24	125	68
	91	39	47	2290	1915
	92	10	15	116	65
	93	24	26	135	141
	94	25	58	164	207
	95	10	41	288	230
	96	20	67	228	131
Mean		22	50	400	298
Counts		12	12	12	12
SD		18	62	616	516
SE		5	18	178	149

Adjuvant	Animal No.	Anti-foetal calf serum IgG titre (x1000)			
		2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
CF/ICF	145	6	23	246	130
	146	4	42	173	59
	147	9	143	395	164
	148	4	29	156	45
	149	15	332	1453	195
	150	15	77	1015	317
	151	32	277	990	806
	152	9	141	855	636
	153	12	97	676	473
	154	22	289	1837	496
	155	7	84	301	173
	156	9	97	994	382
Mean		12	136	758	323
Counts		12	12	12	12
SD		8	106	535	240
SE		2	31	154	69

Example 8

In this example the immunogen was ovalbumin. It was injected into rats in complete Freund's adjuvant for the primary injection and incomplete Freund's for a booster injection. The antibody response obtained was compared with that of sheep given the same antigen formulated in an adjuvant prepared according to this invention.

Female Long-Evans hooded rats were kept in cages of 4 rats/cage. Twenty five, twelve weeks old Long-Evans rats were weighed and divided into two groups. 0.2 to .5 ml blood was collected from tail vein prior to vaccination. For the primary injection using an adjuvant formulation typical of this invention, 65 µg ovalbumin, 40 µg Quil A and 0.8 mg DEAE-dextran were dissolved in 0.128 ml of phosphate buffered saline and emulsified with 0.192 ml of Montanide 888 oil (60 %). In the comparison group 65 µg ovalbumin was dissolved in 0.16 ml of phosphate buffered saline and emulsified with 0.16 ml of Complete Freund's adjuvant (50 %). Both groups were injected subcutaneously as a total volume of 0.32 ml divided into two sites at the back.

Two weeks after the primary injection the rats were weighed, tissue reactions at the injection sites were inspected and 0.2 to 0.5 ml blood was collected from tail vein.

Twelve days later the rats were re-weighed, tissue reactions at the injection sites were inspected and 0.2 to 0.5 ml blood was collected from tail vein. For the booster injection, 65 µg ovalbumin was given subcutaneously as in primary injection. Incomplete Freund's Adjuvant was used in place of complete Freund's adjuvant for the comparison group.

One week after the booster injection and then again two weeks after the booster injection the rats were weighed again, tissue reactions at the injection sites were inspected and 0.2 to 0.5 ml blood was collected from tail

vein.

Four weeks after the booster injection the rats were weighed and tissue reactions at the injection sites were inspected.

Eight weeks after the booster injection the rats were weighed, tissue reactions at the injection sites were inspected and 0.2 to 0.5 ml blood was collected from tail vein.

ELISA on 50 ng ovalbumin/well coated microtitre plates

Ovalbumin was diluted as 50 ng/0.1 ml in carbonate coating buffer pH 9.6 and 100 µl of the solution was added to all wells of row 2 to 12 of 96 wells, flat bottom, microtitre plates (Nunc-Immuno plate, F 96 Cert.maxisorp, Cat. 439454). After overnight incubation at 4°C, the plates were washed four times with 0.05 % tween 20 in saline. After the washing, 100 µl of 0.1 % gelatine in phosphate buffered saline was added to all wells of microtiter plates. This was followed by the addition of the reference serum and sera for testing, diluted 1/100 in 0.1 % gelatine in phosphate buffered saline, to the wells of row 2. Two fold serial dilutions were performed across the plates. After 2 hours incubation at room temperature, the plates were washed four times and 100 µl of 1/16,000 anti-rat IgG, developed in goat, conjugated to peroxidase (Sigma Cat. A 9037) was added and incubated for a further 2 hours. After washing the plate four times 3, 3', 5, 5'-tetramethylbenzidine (Sigma Cat. T2885) substrate was added and incubated for a further 20 minutes before the stopping solution was added. Titres were expressed as the reciprocal of the dilution resulting in 0.5 optical density of the wells.

The results are shown in Figure 7 and Tables 16 to 18.

Table 16. Adjuvant trial in Long-Evans female rats

Adjuvant	Cage/ Animal No.	Body weight (gm)						
		Before prime	2 weeks after prime	Before boost 1	1 week after boost 1	2 weeks after boost 1	4 weeks after boost 1	8 weeks after boost1
(Quil A/ DEAE/ 888 oil)	Cage1/0	197	208	212	214	219	224	222
	Cage1/1	199	212	220	210	237	234	230
	Cage1/2	182	189	196	192	198	202	204
	Cage1/3	203	201	208	216	234	217	222
	Cage 2/0	192	207	217	215	220	203	232
	Cage 2/1	207	222	226	221	228	229	233
	Cage 2/2	202	211	214	215	222	225	226
	Cage 2/3	234	224	230	231	234	241	247
	Cage 3/1	189	170	198	219	232	215	211
	Cage 3/2	205	190	230	240	233	221	234
	Cage 3/3	198	184	207	211	213	238	234
	Cage 7/1	223	224	223	233	234	232	235
	Cage 7/2	228	229	227	238	238	235	235
Mean		205	205	216	220	226	224	228
Counts		13	13	13	13	13	13	13
SD		15	18	11	13	12	12	11
SE		4	5	3	4	3	3	3

Adjuvant	Cage/ Animal No.	Body weight (gm)						
		Before prime	2 weeks after prime	Before boost 1	1 week after boost 1	2 weeks after boost 1	4 weeks after boost 1	8 weeks after boost1
CF/ICF	Cage 4/0	205	214	219	222	222	231	246
	Cage 4/1	186	198	209	204	207	221	222
	Cage 4/2	198	207	206	206	210	220	225
	Cage 4/3	200	211	230	224	227	233	236
	Cage 5/0	198	216	220	222	232	233	235
	Cage 5/1	194	208	210	206	218	229	221
	Cage 5/2	196	206	213	215	222	226	230
	Cage 5/3	205	213	218	224	234	237	241
	Cage 6/0	191	204	221	218	221	225	253
	Cage 6/1	207	223	254	233	236	235	240
	Cage 6/2	215	234	241	236	241	240	248
	Cage 6/3	182	203	212	208	210	210	212
Mean		198	211	221	218	223	228	234
Counts		12	12	12	12	12	12	12
SD		9	10	14	11	11	8	12
SE		3	3	4	3	3	2	4

Table 17. Adjuvant trial in Long-Evans female rats

Adjuvant	Cage/ Animal No.	5 days after prime	11 days after prime	1 week after boost 1	2 weeks after boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	Cage1/0					
	Cage1/1					1+
	Cage1/2					1+
	Cage1/3				1++	1+
	Cage 2/0					1+
	Cage 2/1					
	Cage 2/2	1 +++	1++			1+
	Cage 2/3					1+
	Cage 3/1					
	Cage 3/2					1+
	Cage 3/3					
	Cage 7/1					
	Cage 7/2					

Adjuvant	Cage/ Animal No.	5 days after prime	11 days after prime	1 week after boost 1	2 weeks after boost 1	8 weeks after boost 1
CF/ICF	Cage 4/0	1 ++	1 ++		1 +	2 ++
	Cage 4/1	1 +	1 +		1 +++	2 ++
	Cage 4/2	1 ++	1 ++		1 ++	2 ++
	Cage 4/3	1 +	1 +		2 ++	2 ++
	Cage 5/0	1 ++	1 ++		2 ++	2 ++
	Cage 5/1	2 ++	2 ++		2 +	1 +, 1 ++
	Cage 5/2	1 +	1 +		1 +, 1 +++	1 +, 1 ++
	Cage 5/3		1 +			1 +, 1 +++
	Cage 6/0	1 +++ opened	1 ++	2 +	2 +	1 +, 1 ++
	Cage 6/1	1 ++ opened	1 ++	1 +++ opened	1 +++	2 +
	Cage 6/2		1 +	1 +++	1 +, 1 +++	1 +, 1 +++
	Cage 6/3	1 ++	1 ++		2 ++	2 ++

+ = small lump (1-2 mm) ++ = Medium lump (3-5 mm) +++ = Big lump (more than 5 mm)
1 and 2 = numbers of lumps

Table 18. Adjuvant trial in Long-Evans female rats

Adjuvant	Cage/ Animal No.	Titre (x1000)			
		2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
(Quil A/ DEAE/ 888 oil)	Cage1/0	1	33	155	247
	Cage1/1	1	38	68	156
	Cage1/2	1	13	28	96
	Cage1/3	0	9	29	77
	Cage 2/0	1	36	93	126
	Cage 2/1	1	26	85	92
	Cage 2/2	0	12	23	49
	Cage 2/3	0	33	78	173
	Cage 3/1	2	55	155	68
	Cage 3/2	0	28	68	195
	Cage 3/3	10	91	164	246
	Cage 7/1	1	57	210	252
	Cage 7/2	0	21	80	150
Mean		1	35	95	148
Counts		13	13	13	13
SD		3	22	59	71
SE		1	6	16	20

Adjuvant	Cage/ Animal No.	Titre (x1000)			
		2 weeks after prime	Before boost 1	2 weeks after boost 1	8 weeks after boost 1
CF/ICF	Cage 4/0	0	18	94	57
	Cage 4/1	0	12	38	33
	Cage 4/2	4	63	120	81
	Cage 4/3	4	67	98	66
	Cage 5/0	2	39	113	69
	Cage 5/1	2	46	67	65
	Cage 5/2	1	2	27	34
	Cage 5/3	2	68	87	55
	Cage 6/0	11	89	174	128
	Cage 6/1	0	62	126	87
	Cage 6/2	4	7	27	36
	Cage 6/3	3	66	95	87
Mean		3	45	89	67
Counts		12	12	12	12
SD		3	29	44	27
SE		1	8	13	8

Examples 5 to 8 demonstrate that the newly invented adjuvant stimulates the immune system of a variety of animal species against a range of antigens with an efficacy similar to or better than the benchmark Freund's adjuvant but without the injection site reactions induced by the latter.

INDUSTRIAL APPLICABILITY

The adjuvant compositions of the present invention are applicable to the preparation of vaccines against a wide range of infectious diseases and against natural products of the human and animal body such as hormones.

CLAIMS

1. An adjuvant composition for stimulating an effective immune response to an antigenic substance when co-administered to an animal with said antigenic substance, comprising:
   (a) a saponin with immune stimulating activity;
   (b) a polycationic polyelectrolyte with immune stimulating activity; and
   (c) an immunoadjuvant oil.
2. An adjuvant composition as claimed in claim 1 wherein the saponin is a triterpenoid compound or a mixture of triterpenoid compounds.
3. An adjuvant composition as claimed in claim 2 wherein the saponin is Quil A.
4. An adjuvant composition as claimed in any one of claims 1 to 3 wherein the polycationic polyelectrolyte is diethylaminoethyl dextran.
5. An adjuvant composition as claimed in any one of claims 1 to 4 wherein the immunoadjuvant oil is a mineral oil.
6. An adjuvant composition as claimed in claim 5 wherein the mineral oil is Freund's incomplete adjuvant or a Montanide oil.
7. A vaccine for administration to an animal, comprising:
   (1) an antigenic substance; and
   (2) an adjuvant composition comprising:
      (a) a saponin with immune stimulating activity;
      (b) a polycationic polyelectrolyte with immune stimulating activity;
      (c) an immunoadjuvant oil.
8. A vaccine as claimed in claim 7 wherein the saponin is a triterpenoid compound or a mixture of triterpenoid compounds.
9. A vaccine as claimed in claim 8 wherein the saponin is Quil A.
10. A vaccine as claimed in any one of claims 7 to 9 wherein the polycationic polyelectrolyte is diethylaminoethyl dextran.

11. A vaccine as claimed in any one of claims 7 to 10 wherein the immunoadjuvant oil is a mineral oil.

12. A vaccine as claimed in claim 11 wherein the mineral oil is Freund's incomplete adjuvant or a Montanide oil.

13. A vaccine according to any one of claims 7 to 13 wherein the saponin is present in a concentration of between 50µm/ml and 10mg/ml.

14. A vaccine according to claim 13 wherein the saponin is present in a concentration between 100µm/ml and 1mg/ml.

15. A vaccine as claimed in any one of claims 7 to 14 wherein the polycationic polyelectrolyte is present in a concentration between 1mg/ml and 200mg/ml.

16. A vaccine as claimed in claim 15 wherein the polycationic polyelectrolyte is present in a concentration between 1.5mg/ml and 150mg/ml.

17. A method of stimulating an effective immune response in an animal to an antigenic substance, comprising the steps of:
    (1) providing said antigenic substance;
    (2) providing an adjuvant composition comprising:
        (a) a saponin with immune stimulating activity;
        (b) a polycationic polyelectrolyte with immune stimulating activity; and
        (c) an immunoadjuvant oil; and
    (3) challenging said animal with said antigenic substance and said adjuvant composition.

18. The use of an adjuvant composition comprising:
    (a) a saponin with immune stimulating activity;
    (b) a polycationic polyelectrolyte with immune stimulating activity; and
    (c) an immunoadjuvant oil
    to stimulate an effective immune response in an animal challenged with an antigenic substance.

19. The use of an adjuvant composition comprising:
    (a) a saponin with immune stimulating activity;

(b) a polycationic polyelectrolyte with immune stimulating activity; and

(c) an immunoadjuvant oil

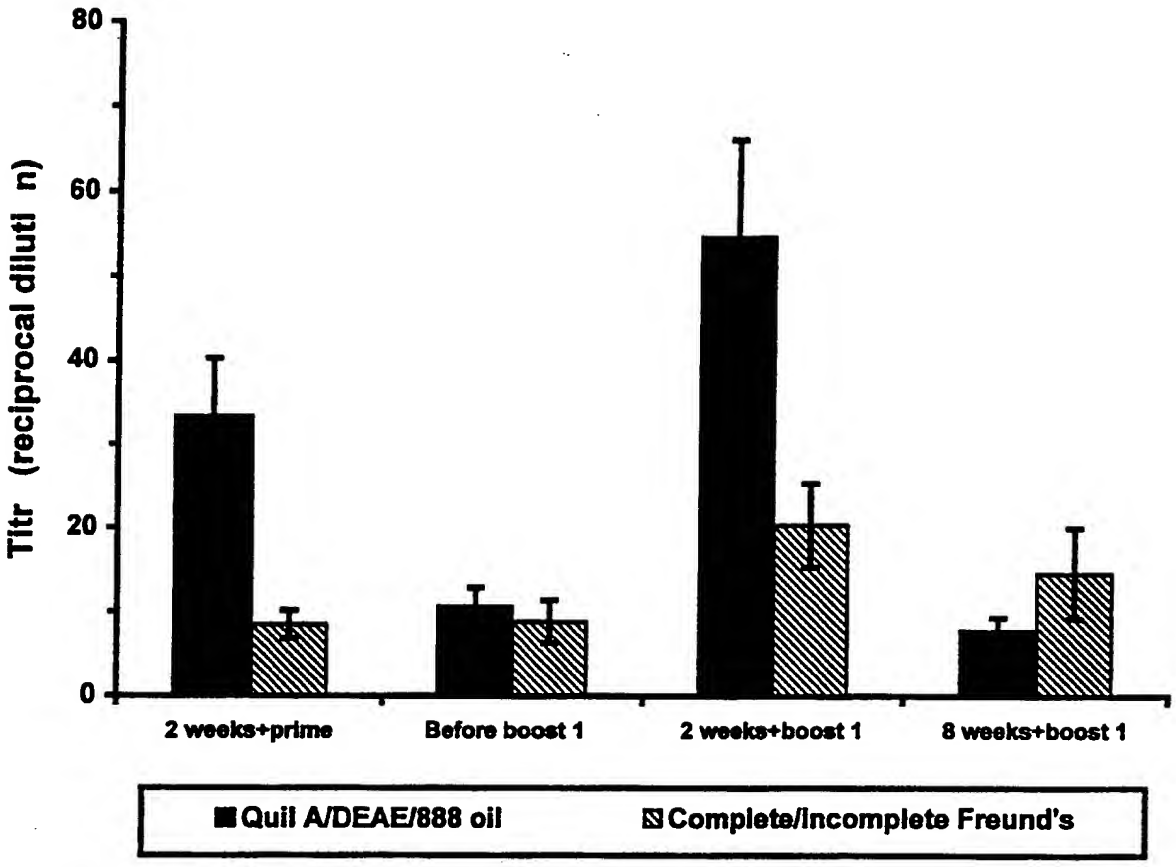
in the preparation of a medicament for administration to an animal, wherein said medicament further comprises an antigenic substance.

20. An adjuvant composition substantially as hereinbefore described with reference to the Examples.

21. A vaccine substantially as hereinbefore described with reference to the Examples.

22. A method of vaccinating animals substantially as herein described with reference to the Examples.

Figur 1.


Titr (reciprocal dilutl n)
80
60
40
20
0
2 weeks+prime
Before boost 1
2 weeks+boost 1
8 weeks+boost 1
Quil A/DEAE/888 oil
Complete/Incomplete Freund's

Figur 2.

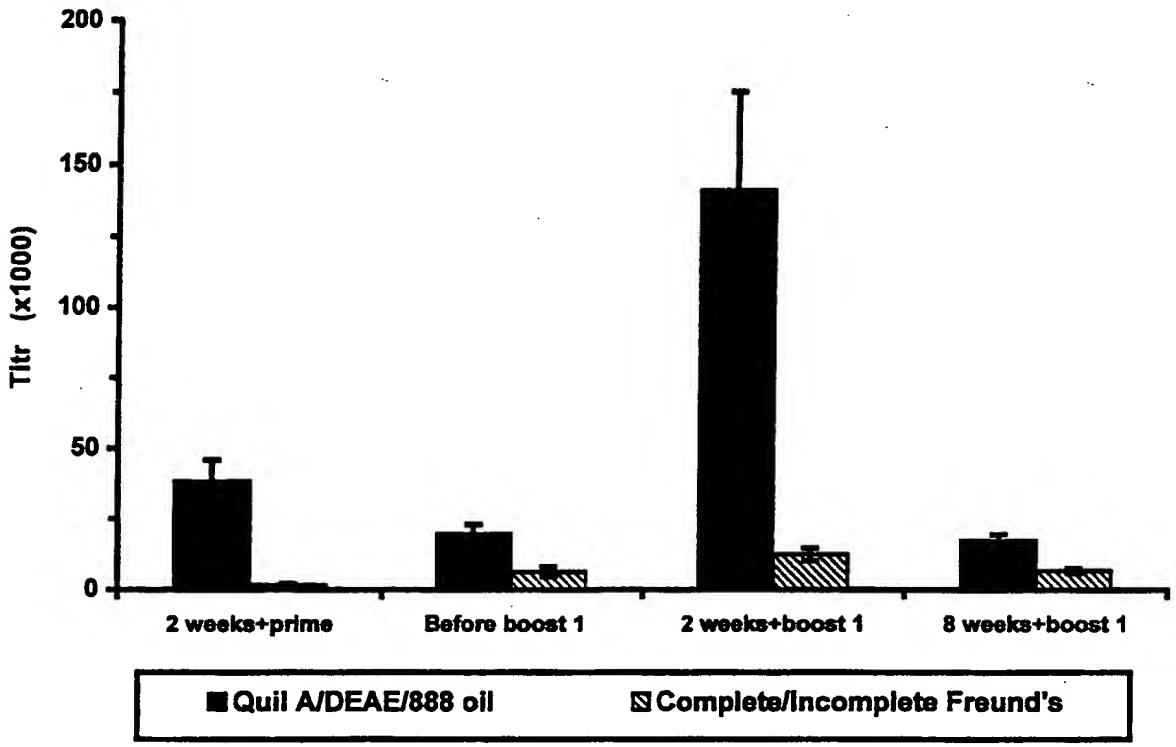
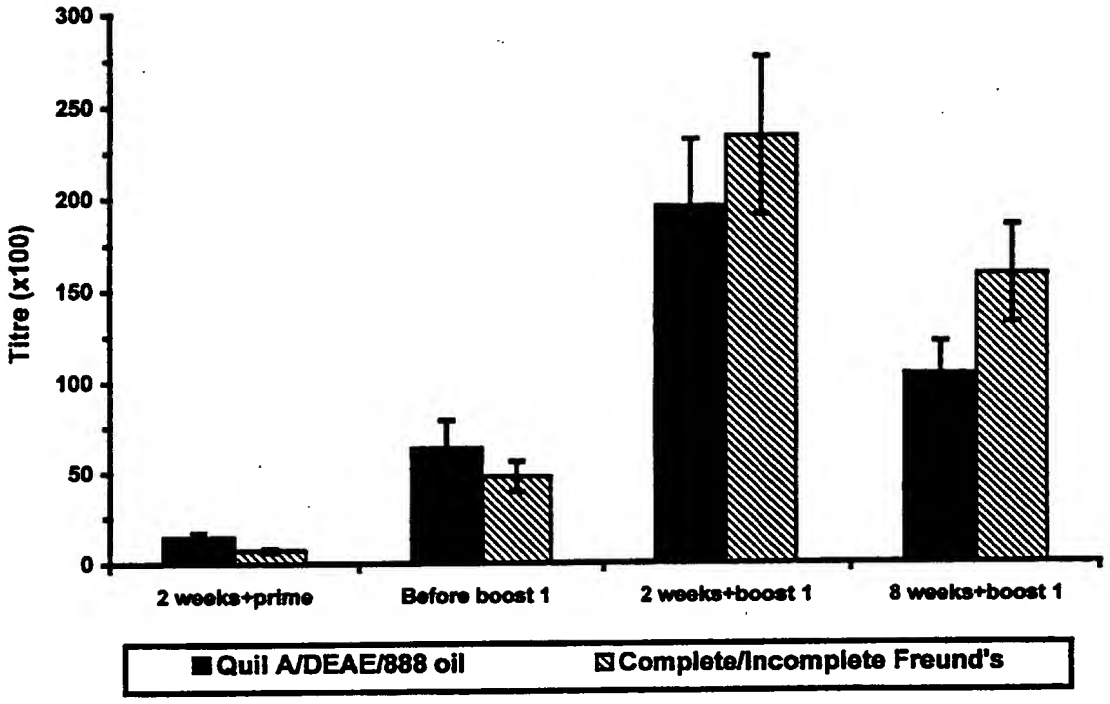

200
150
100
50
0
Titr (x1000)
2 weeks+prime
Before boost 1
2 weeks+boost 1
8 weeks+boost 1
Quil A/DEAE/888 oil
Complete/Incomplete Freund's

Figure 3.


300
250
200
150
100
50
0
Titre (x100)
2 weeks+prime
Before boost 1
2 weeks+boost 1
8 weeks+boost 1
Quil A/DEAE/888 oil
Complete/Incomplete Freund's

Figur 4.

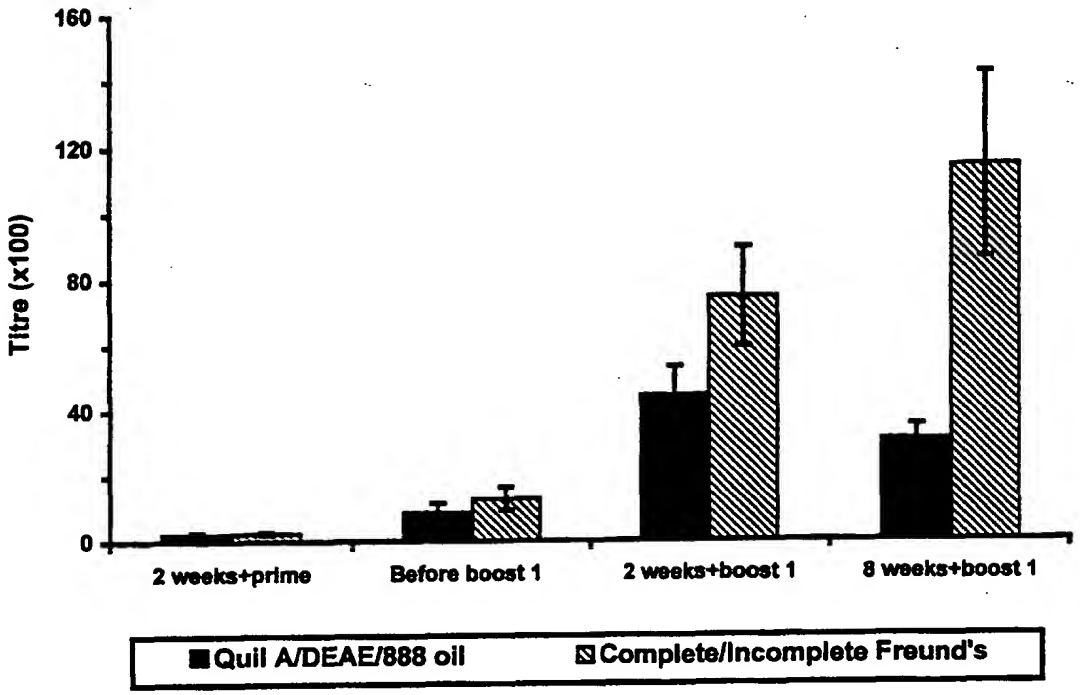

160
120
80
40
0
Titre (x100)
2 weeks+prime
Before boost 1
2 weeks+boost 1
8 weeks+boost 1
Quil A/DEAE/888 oil
Complete/Incomplete Freund's

Figure 5.

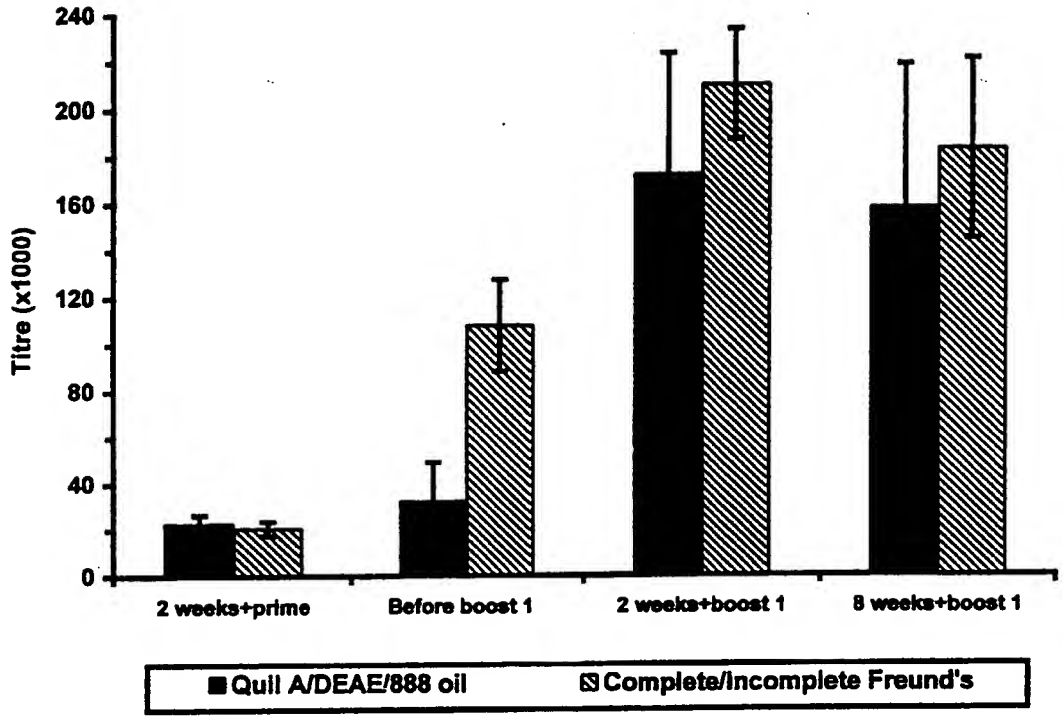

240
200
160
120
80
40
0
Titre (x1000)
2 weeks+prime
Before boost 1
2 weeks+boost 1
8 weeks+boost 1
Quil A/DEAE/888 oil
Complete/Incomplete Freund's

Figure 6.

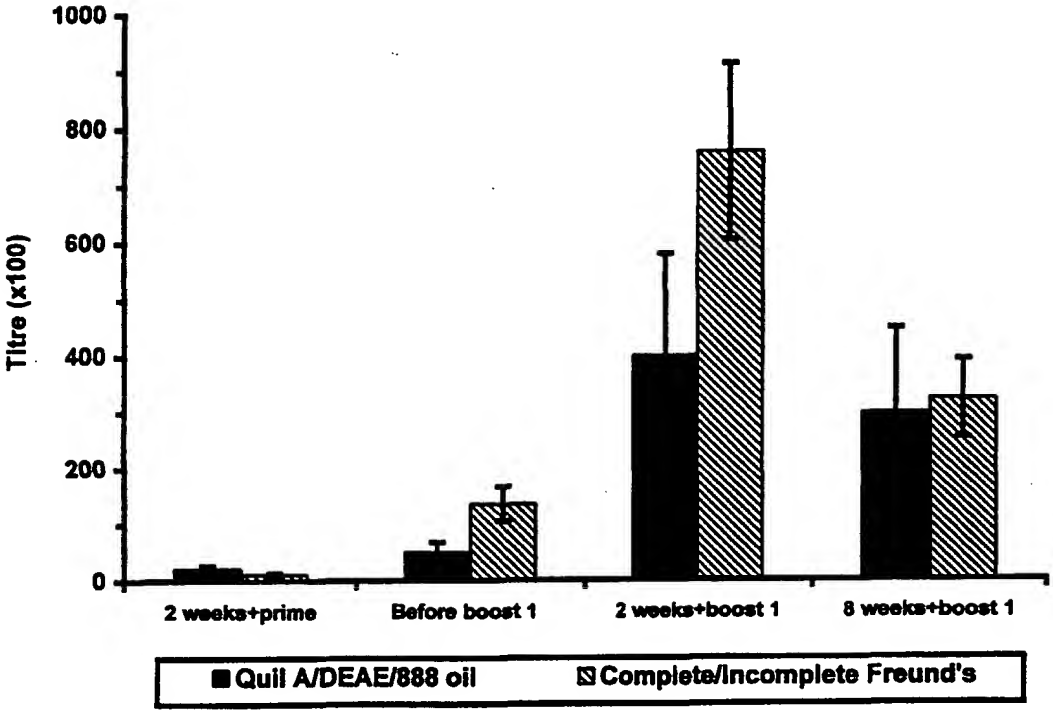
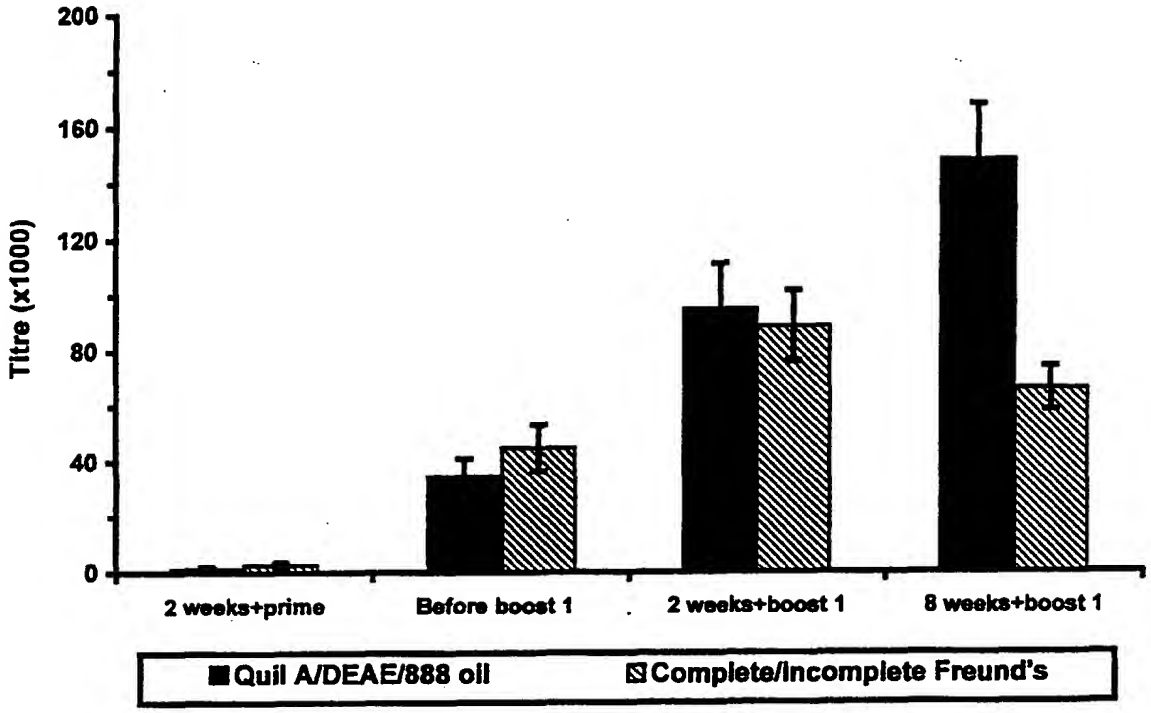

1000
800
600
400
200
0
Titre (x100)
2 weeks+prime
Before boost 1
2 weeks+boost 1
8 weeks+boost 1
Quil A/DEAE/888 oil
Complete/Incomplete Freund's

Figure 7.


200
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40
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Titre (x1000)
2 weeks+prime
Before boost 1
2 weeks+boost 1
8 weeks+boost 1
Quil A/DEAE/888 oil
Complete/Incomplete Freund's

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 98/0990

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A61K 039/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC A61K-039

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, MEDLINE, CAPLUS, USPTO: Saponin* or Quil*

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 91/04052 A (PEPTIDE TECHNOLOGY LIMITED) 4 April 1991 Entire document	1-22
A	WO 88/07547 A (COOPERS ANIMAL HEALTH LIMITED) 6 October 1988 Entire document	1-22
A	EP 242205 A2 (NORDEN LABORATORIES, INC) 21 October 1987 Entire document	1-22

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 December 1998	08 JAN 1999
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer T. SUMMERS Telephone No.: (02) 6283 2291

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 98/0990

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5688772 A (ESTRADA et al.) 18 November 1997 Entire document	1-22
A	Estuningsih SE, "Evaluation of Antigens of *Fasciola gigantica* as Vaccine Against Tropical Fasciolosis in Cattle, *Int. J. Parasitol.*, Nov 1997, 27(11), 1419-28 Entire document	1-22
A	Medline Abstract Accession No. 85301845, Vanselow BA, *Vet. Rec.* 13 July 1985, 117(2) 37-43 Entire document	1-22
A	Medline Abstract Accession No. 92348094, East IJ, *Int. J. Parasitol.*, May 1992, 22(3), 309-14 Entire document	1-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application N .
PCT/AU 98/0990

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9104052	AU	64210/90	CA	2066653	EP	491833
WO	8807547	AU	14968/88	BR	8806474	CA	1330420
		DK	6618/88	EP	284406	HU	52787
		NZ	224028	PT	87080	US	5401829
		ZA	8802166				
EP	242205	AU	71760/87	CA	1282003	DK	1903/87
		JP	62255436	PT	84692	US	4806350
		ZA	8702743				
US	5688772	AU	33384/95	CA	2196082	EP	773786
		US	5597807	WO	9603998		

END OF ANNEX